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Research Article

Melatonin Ameliorates Cyclophosphamide-Induced Spermatogenesis Disorder by Reducing Pyroptosis

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As a chemotherapeutic drug, cyclophosphamide (CP) has a negative impact on male fertility due to its reproductive toxicity. Melatonin (Mel) promotes the male reproductive system and increases testosterone synthesis. This study is aimed at exploring the molecular mechanism of Mel as a protector of male fertility against CP-induced cytotoxicity. A CP toxicity model was established in adult ICR male mice by intraperitoneal injection of 100 mg/kg CP every other day for a week. Protective effects of Mel on the testis from CP-induced damage were evaluated using four groups of ICR male mice that received intraperitoneal injections of normal saline, 100 mg/kg CP, 10 mg/kg Mel, or the same dosage of CP and Mel, respectively. Testis morphology was observed by hematoxylin and eosin (HE) staining. Sperm quality parameters were evaluated, and sperm function was studied by in vitro fertilization (IVF). Proliferation, meiosis, and pyroptosis markers were examined by western blot. Results showed that CP treatment induced testis toxicity in a time-dependent manner with the most severe damage to the testis at two weeks post CP treatment. CP-treated mice showed reduced testicular weight and impaired spermatogenesis by downregulating PCNA and SYCP3, reduced serum testosterone levels, decreased sperm counts and motility, increased seminiferous tubule vacuolization, and oxidative damage to spermatogenic cells. All these effects, apart from testicular weight, could be ameliorated by Mel administration. The IVF experiment revealed that CP treatment reduced the rates of sperm fertilization and blastocyst development, which were also enhanced by Mel. Mel-treated mice also showed increased expression of proliferation-associated protein PCNA and meiosis-associated proteins REC8, STRA8, and SYCP3, which were all reduced by CP. Furthermore, Mel inhibited the pyroptosis of spermatogenic cells by reducing GSDMD and IL18 expression. In conclusion, this study indicated that Mel might protect the testis from CP-induced DNA damage to germ cells through the alleviation of pyroptosis.

1. Introduction

Cancer therapies, especially chemotherapy, are known to cause male reproductive toxicity. Due to the changes in spermatogenesis caused by chemotherapy drugs, many men are permanently infertile. Cyclophosphamide (CP), which alkylates DNA and creates cross-links, is frequently used in cancer treatment and as an antagonist of rejection in transplant patients. Despite having several clinical applications, CP causes cytotoxicity in human and experimental animals. Many studies have reported that exposure to CP among humans and animals induced toxicity to various organs, including the heart, liver, lung, testis, and ovary [1–3].

Generally, the cytotoxicity of alkylating drugs is caused by necrosis, apoptosis, pyroptosis, or autophagy, depending on the cell morphology and cellular signaling pathway. Previous studies have focused on the activation of CP-induced inflammation, oxidative stress, and apoptosis. Oxidative stress and apoptosis induction are the known mechanisms that CP causes toxicity in the male reproductive system [4, 5]. Recent research suggested that inflammation and pyroptosis play important roles in spermatogenesis dysfunction. Pyroptosis is a programmed cell death accompanied with an inflammatory response, which disrupts the integrity of cell membranes and results in the fluxion of a large number of substances. With the release of proinflammatory agents,

pyroptosis enlarges secondary inflammation. CP was demonstrated to impair the function of bladder muscle, ovary, and hepatic tissues by inducing pyroptosis [6–8]. The levels of gasdermin D (GSDMD), caspase-1, interleukin 18 (IL18), and interleukin 1β (IL1β) were significantly elevated after CP treatment [6, 7]. Therefore, we hypothesized that the cytotoxicity of CP on the testis may be due to a pyroptosis-inducing effect.

Due to the adverse effects of CP on the testis, it is necessary and urgent to develop and applicate a cytoprotector. The introduction of substances with antioxidant characteristics might either counteract or prevent the prooxidative stress impact of CP. It has been reported that some natural compounds such as astaxanthin, melatonin, tribulus terrestris, and crocin could protect the testes from CP-induced oxidative stress damage [9–12].

Melatonin (N-acetyl-5-methoxy-tryptamine, Mel) is an endogenous hormone secreted mainly by the pineal gland and synthesized in reproductive organs such as the ovary and testis [13]. It carries out a variety of tasks, including controlling metabolism, apoptosis, and inflammation [14, 15]. Recently, the role of Mel in male infertility has attracted extensive attention. Mel participates in male reproduction and spermatogenic cell proliferation by regulating the secretion of steroid hormones [16, 17]. In addition, due to its lipophilic and hydrophilic free radical-scavenging properties, Mel protects the testis from environmental toxins, hyperpyrexia, and drug-induced damage [18, 19].

There were three studies showing that Mel prevented testicular toxicity induced by CP in mice and rats. CP caused irregular seminiferous tubules and perivascular fibrosis and decreased the thickness of epithelial layers. Pretreatment of Mel prevented CP-induced spermatogenesis toxicity by increasing glutathione levels, glutathione peroxidase, and alkaline phosphatase activity, decreasing malondialdehyde level, the activity of superoxide dismutase and catalase in testes, and increasing plasma testosterone level [10, 20, 21]. However, the impairment of male fertility caused by CP treatment and the protective effect of Mel on male fertility have not been studied, and the molecular mechanism of Mel other than the antioxidative property remains unclear. Therefore, this study is aimed at exploring the effect of Mel on ameliorating sperm quality and spermatogenesis and the possible regulation of the pyroptosis pathway.

In this research, we studied whether Mel has a protective effect against CP-induced changes in sperm fertilization rate and embryo development and the effect of Mel on spermatogenic cell proliferation, meiosis, and pyroptosis. Furthermore, we sought to identify the underlying mechanisms involved in this cellular protective effect.

2. Materials and Methods

2.1. Animals. All animal experiment protocols were approved by the Animal Ethics Committee of Yantai YuHuangDing Hospital (No. 2021-163). All animal experiments complied with the Guide for the Care and Use of Laboratory Animals. Institute of Cancer Research (ICR) mice were procured from Beijing Vital River Laboratory Animal Technology Co., Ltd., China. The animals were raised at room temperature and appropriate humidity with a cycle of 12 hours of light and 12 hours of darkness. Standard laboratory animal feed and water were provided.

2.2. Animal Experiment. Forty male ICR mice (6-8 weeks) weighing 30-35 g were randomly divided into control (CN) and CP groups of 20 mice each. The CP group was injected intraperitoneally (i.p.) with CP (100 mg/kg bodyweight, Baxter, Halle, Germany) every other day for a week. The CN mice were i.p. injected with the same dose of normal saline at the same time. After the last injection, five mice from each group were sacrificed at week 1, week 2, week 4, and week 6, respectively. The mice were deeply anesthetized with 20 ml/kg bodyweight of 1.25% Avertin (2,2,2-tribromoethanol, Nanjing AiBei Biotechnology Co., Ltd., China) and euthanized by cervical dislocation. The testes and epididymis of mice were quickly dissected out and weighted. The relative weight of the testis and epididymis was expressed as a percentage of body weight. All testes were snap-frozen in liquid nitrogen and utilized for protein extraction.

Thirty-two ICR male mice were used to evaluate the protective effect of Mel after treatment with CP for two weeks. The ICR male mice were randomly classified into four groups of 8 mice each: the CN group, CP group, Mel group, and CP+Mel group. The CN and CP groups were developed as above. The Mel group was i.p. injected with Mel (10 mg/kg bodyweight, Sigma-Aldrich, St. Louis, USA) every other day for four weeks. The CP+Mel group was i.p. injected with CP for a week and cotreated with Mel starting one week before the first CP injection and continuously given every other day for four weeks. The dose of 10 mg/kg of Mel has previously been shown to have an inhibitory effect against cisplatin- or busulfan-induced testicular dysfunction [19, 22]. After euthanization, one testis was fixed in Bouin's solution for histological evaluation. The other testis was snap-frozen in liquid nitrogen and stored at -80°C for extraction of RNA and protein.

The number of mice used in the studies was determined by the following formula: total number of animals = degree of freedom of variance analysis (E) + number of groups, in which E should be between 10 and 20. Considering the large individual differences of ICR mice and an estimated loss rate of 20%, the number of animals used was $(20 + 8) \times 120\% = 34$ for the study with two groups and four timepoints, resulting in 5 mice from each group; $(20 + 4) \times 120\% = 29$ for the study with four groups, resulting in 8 mice from each group.

2.3. Assessment of Testosterone. The mice were deeply anesthetized, and then the blood was quickly collected by cardiac puncture after euthanization. After blood coagulation, the serum was separated by centrifugation at $4500 \times g$ for 15 min. Serum testosterone level was analyzed using a Testosterone ELISA Kit (Cayman Chemical, MI, USA) following the manufacturer's protocol. The testosterone standards were prepared by serial dilution using ELISA buffer. Each sample was assayed at a minimum of two dilutions, and each dilution was assayed in triplicate. For each well, 50 μl of standard or sample, 50 μl of AChE tracer, and

50 μl of testosterone ELISA antiserum were added. After incubation for 2 h at room temperature, the wells were rinsed with wash buffer. The reactions were carried out by adding 200 μl of Ellman's reagent. After incubation using an orbital shaker for 1 h in the dark, optical density (OD) at 414 nm was measured. The concentration of testosterone in each sample was calculated using the equation obtained from the standard curve plot.

2.4. Sperm Count and Sperm Quality Analysis. The cauda epididymis and vas deferens of mice were cut into small pieces in PBS containing 10% (*w*/*v*) bovine serum albumin (BSA, Biotopped, Beijing, China). The tissue was incubated at 37°C for 5 min to allow the sperm to swim out. The released spermatozoa were collected for analyzing sperm parameters using a computer-aided semen analysis system (CASA) (Medealab™, Erlangen, Germany), including sperm concentration, sperm motility, progressive sperm ratio, straight-line velocity (VSL), curvilinear velocity (VCL), and average path velocity (VAP). The spermatozoa were counted across 10 different fields of view per slide. There were about 500 to 1000 spermatozoa taken into account for each sample.

2.5. In Vitro Fertilization (IVF). An IVF experiment was performed to assess male mouse fertility and fecundity. The sperm of cauda epididymidis were collected and incubated in 1 ml of modified Krebs-Ringer bicarbonate medium (TYH) for 1 h for sperm capacitation. Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) of 10 i.u. each were used in superovulation by i.p. injection at 48 h intervals. Fourteen hours after injection of hCG, female mice were euthanized under anesthesia, and cumulus oocyte complexes (COCs) were isolated from the ampullary segment of the oviduct using pointed tweezers. After being washed in human tubal fluid (HTF) medium, COCs were then introduced into each sperm suspension of 10^6 spermatozoa/ml for insemination. The sperm from every two male mice within each group were mixed and incubated with approximately 100 oocytes isolated from 3 female mice. After 4-6 hours of sperm-egg incubation, embryos were washed in M2 medium to remove cumulus cells and placed in balanced KSOM medium for the following culture. The morphology of embryonic development was observed every day. The reagents used in the IVF experiment were purchased from the Nanjing Aibei Biotechnology Co., Ltd. (China). Male fertility was measured by the sperm fertilization rate and the embryo development rate. The fertilization rate was defined as the percentage of fertilized eggs out of total oocytes. Two-cell embryos' development rate was calculated as the number of two-cell embryos/the number of fertilized eggs, and blastocysts' development rate was calculated as the number of blastocysts/the number of two-cell embryos.

2.6. HE Staining and Immunohistochemistry (IHC). Fixed testes were dehydrated in graded ethanol and embedded in paraffin. The 4 μm tissue sections were then deparaffinized and rehydrated. Sections were stained with HE for histological studies. The slides were observed and photographed by bright field microscopy (DM LB2, Leica, Nussloch, Germany). ImageJ software was used for image analysis. The thickness of seminiferous epithelium was calculated as (the diameter of seminiferous tubule - the diameter of tubule lumen) / 2. At least thirty seminiferous tubules were measured for each group. The number of Leydig cells in interstitial space was calculated as the number of Leydig cells per interstitial compartment/the area of the interstitial compartment. Twenty interstitial compartments were measured and quantified for each group.

IHC was performed as previously described [3] using the primary rabbit antibody against proliferating cell nuclear antigen (PCNA, 1: 1000, D220014, Sangon, Shanghai, China) and horseradish peroxidase- (HRP-) conjugated IgG antibody (1: 400, ZB-2301, Zhong-Shan Biotechnology, Beijing, China). The slides were visualized by bright field microscopy (DM LB2, Leica, Nussloch, Germany). The positive stains were dark brown in color and mainly distributed in the cell nucleus. The positive cells of each seminiferous tubule were counted. At least 10 fields per section of view were randomly selected for statistical analysis.

2.7. Western Blot. The testes were homogenized and lysed using RIPA lysis buffer (Beyotime, Shanghai, China), supplemented with a cocktail of protease inhibitors (Beyotime, Shanghai, China). Protein concentration was determined using the BCA Protein Assay Kit (Beyotime, China). The different protein samples of each group were mixed. Western blot was routinely performed using primary rabbit antibodies raised against the following antigens: oxidative stress-associated proteins, including superoxide dismutase 1 (SOD1, 1 : 1000, AF8028, Beyotime), glutathione peroxidase 1 (GPX1, 1 : 1000, GTX116040, GeneTex), catalase (CAT, 1 : 1000, D122036, Sangon), and peroxiredoxin 1 (PRDX1, 1 : 1000, ab109498, Abcam); proliferation marker PCNA (1 : 1000, D220014, Sangon); meiosis markers, including REC8 meiotic recombination protein (REC8, 1 : 1000, D222997, Sangon), stimulated by retinoic acid 8 (STRA8, 1 : 1000, D261151, Sangon), and synaptonemal complex protein 3 (SYCP3, 1 : 1000, ab15093, Abcam); pyroptosis-associated protein, including GSDMD (1 : 1000, ab219800, Abcam) and IL18 (1 : 1000, ab71495, Abcam). HRP-conjugated anti-IgG was used as the secondary antibody. An enhanced chemiluminescence (ECL) kit (Vazyme, Nanjing, China) was used to detect the immune-reactive signals. The protein bands were quantified by ImageJ software using GAPDH (1 : 3000, D110016, Sangon) and ubulin (1 : 3000, D191046, Sangon) as the loading control.

2.8. RNA Isolation and Real-Time Quantitative PCR (qPCR). The expression of pyroptosis-related genes in mouse testes was quantified using qPCR with SYBR Green labeling. TRIzol reagent (TaKaRa Biotechnology, Dalian, China) was used to extract total RNA from testis samples. The mRNA levels of target genes were quantified using the SYBRGreen qPCR Master Mix kit (Vazyme, Nanjing, China). *ACTB* was used as the internal control. The primer sequences were as follows: *GSDMD*: forward, 5′-AGTGGCCCAACTGCTTATT-3′, reverse, 5′-CTCCTCATCAATCCCATCTGAC-3′; *GSEME*: forward, 5′-CCTGGAAGATGTACTCACAGA

AG-3′, reverse, 5′-TCAGGGTTCCAAACGAAGAG-3′; *IL18*: forward, 5′-CTTTGGCCGACTTCACTGTA-3′, reverse, 5′-CACAGCCAGTCCTCTTACTTC-3′; *IL1b*: forward, 5′-TCATTGTGGCTGTGGAGAAG-3′, reverse, 5′-GCCTGTAGTGCAGTTGTCTAA-3′; *Casp1*: forward, 5′-CCAGGCAAGCCAAATCTTTATC-3′, reverse, 5′-GGAAATGTGCCATCTTCTTTGT-3′; *ACTB*: forward, 5′-CTCCCTGGAGAAGAGCTATGA-3′, reverse, 5′-GGCATAGAGGTCTTTACGGATG-3′. The linear amount of target molecules compared to the calibrator was estimated by $2^{-\Delta\Delta CT}$.

2.9. Statistical Analysis. GraphPad Prism 8.0 software was used to perform statistical analysis and generate graphs. Numeric data were presented as mean ± standard deviation (SD) and analyzed using the student's *t*-test or one-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. CP Treatment Reduced Mouse Testis Weights and Impaired Spermatogenesis. We studied the impact of CP on the testis, epididymis, and spermatogenesis of ICR mice at weeks 1, 2, 4, and 6 after CP treatment. Relative testicular weight (testis/body weight) in the CP group was significantly reduced compared to the CN at weeks 2 and 4. Relative epididymis weight (epididymis/body weight) was not significantly different between the CP and CN groups (Figure 1(a)). To explore the effects of CP on spermatogenesis, the expression of proliferation-associated protein PCNA and meiosis-associated protein SYCP3 was analyzed by western blot. As shown in Figure 1(b), the expression of PCNA and SYCP3 in the CP group significantly decreased during six weeks, and the biggest reductions in PCNA and SYCP3 expression compared with the CN group were observed at week 2 (69.7% and 90.6% reduction, respectively).

3.2. Mel Ameliorated CP-Induced Impairment in Serum Testosterone Level and Sperm Quality. Based on the above results, we chose the time point of two weeks after CP treatment to assess whether Mel protects the testis from CP-induced damage. The CP+Mel group mice were pretreated with Mel for one week before CP injection. Two weeks after CP treatment, the blood serum, testis, and sperm among the CN group, CP group, Mel group, and CP+Mel group were collected to assess the function of Mel on testicular weight, testosterone level, and sperm quality. The testicular weight was significantly reduced in CP group and improved in the CP+Mel group compared to the CP group, however with no statistical significance. CP and Mel did not affect epididymal weight (Figure 2(a)). As shown in Figure 2(b), Mel treatment significantly raised the serum testosterone level (14.4 ± 5.7 ng/ml) compared with that in CN group (8.1 ± 1.9 ng/ml). The serum testosterone level was significantly reduced in CP group (2.0 ± 1.4 ng/ml), which was rescued nearly to the level of CN group after Mel administration (7.4 ± 2.8 ng/ml). CP administration significantly reduced sperm concentration ($177.6 \pm 23.0 \times 10^6$/ml), sperm motility (42.1 ± 5.2%), progressive sperm ratio (17.6 ± 4.4%), and sperm movement velocity (including VCL 61.4 ± 5.5 μm/s, VSL 22.0 ± 2.3 μm/s, and VAP 33.8 ± 4.7 μm/s). All these parameters of sperm were restored nearly to the level of the CN group after Mel supplementation, showing sperm concentration of $344.3 \pm 28.2 \times 10^6$/ml, sperm motility of 64.0 ± 5.0%, progressive sperm ratio of 41.6 ± 4.2%, VCL 102.9 ± 9.4 μm/s, VSLof 38.7 ± 3.8 μm/s, and VAP of 59.0 ± 6.0 μm/s (Figure 2(c)).

3.3. Mel Improved Male Mouse Fertility Impaired by CP. Since the sperm quality of mice decreased after CP treatment, we further assessed the changes in sperm function resulting from CP treatment in an IVF experiment. Male mouse sperms of different groups were incubated with normal female oocytes. Results showed that CP treatment decreased the sperm fertilization rate (68.1 ± 5.6%) and blastocyst development rate (37.5 ± 7.5%) but had no effect on the two-cell embryo development rate (78.9 ± 8.8%). Mel treatment restored the impaired sperm function by improving sperm fertilization (79.8 ± 4.1%) and blastocysts development abilities (55.5 ± 4.7%) (Figure 3).

3.4. Mel Protected Testis from Histological Damage and Oxidative Stress Induced by CP. The histopathological changes in the testes were detected by HE staining. As shown in Figure 4(a), administration of Mel alone had no effect on the histological features of the testes. In contrast, CP caused various morphological damages: thinning and loosening of the seminiferous epithelium, degeneration and vacuolization of the seminiferous tubules, and the reduction of interstitial cells. The above histological damage was restored at least to some extent by Mel treatment. There was a regular distribution of spermatogenic cells of different stages, and many mature sperm were distributed in the central of the seminiferous tubules. As shown in Figure 4(b), the thickness of seminiferous epithelium was significantly increased in the CP+Mel group (25.1 ± 5.0 μm) compared with CP group (22.1 ± 5.5 μm), with no statistically significant difference in the diameter of seminiferous tubule between the CP group (81.3 ± 11.5 μm) and the CP+Mel group (80.3 ± 9.9 μm). Besides, the number of Leydig cells in interstitial space increased from $7.8 \pm 3.8 \times 10^{-3}/\mu m^2$ in the CP group to $10.9 \pm 2.1 \times 10^{-3}/\mu m^2$ in the CP+Mel group.

Next, the expressions of oxidative stress markers of different groups were compared, including superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPX1), catalase (CAT), and peroxiredoxin 1 (PRDX1). Quantification of these proteins revealed that the expressions of SOD1, GPX1, CAT, and PRDX1 were dramatically reduced in the CP treatment group to 58.6%, 61.9%, 50.6%, and 51.5% of the CN group, whereas cotreatment with Mel significantly recovered the levels of these proteins to 104.8%, 131.7%, 62.7%, and 62.4% of the CN group, respectively (Figure 4(c)).

3.5. Treatment with Mel Improved Germ Cell Proliferation and Meiosis. To further analyze the protective effect of Mel on the testes, the expression of crucial markers in the proliferation and meiosis of spermatogonia (PCNA, REC8, STRA8, and SYCP3) was measured. The expression of

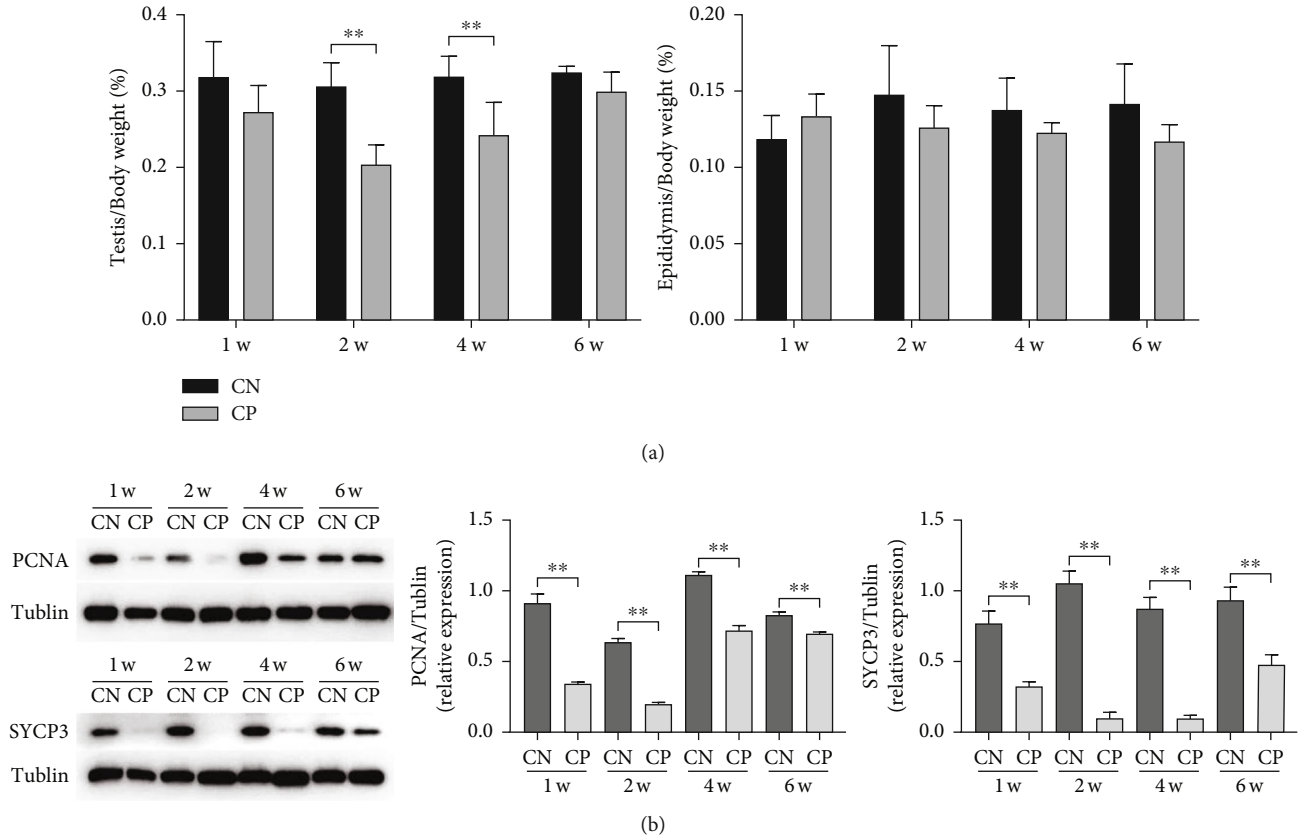

Figure 1: CP-induced damage to the testis of mice. (a) Testicular and epididymal weights during 6 weeks after i.p. injection of CP. (b) The expression of PCNA and SYCP3 in the testis by western blot analysis. All values were shown as mean ± SD and $^{**}p < 0.01$.

PCNA in the testes was analyzed by IHC and western blot. IHC analysis indicated that the positive-staining cells for PCNA were mainly spermatogonia. The number of PCNA-positive cells decreased to 34.8 ± 7.1 per tubule in the CP group compared with 56.5 ± 10.1 per tubule in the CN group, which indicated that CP affected the proliferation of germ cells. The number of PCNA-positive cells was upregulated to 55.7 ± 14.7 per tubule in the CP+Mel group (Figures 5(a) and 5(b)). In addition, western blot analysis of PCNA was in accordance with IHC results (Figures 5(c) and 5(d)). As the key markers of meiosis, the expression of REC8, STRA8, and SYCP3 was analyzed by western blot. The testis expression of REC8, STRA8, and SYCP3 in the CP group was reduced to 31.3%, 35.1%, and 18.3% of the CN group, respectively. The expression of these proteins in CP +Mel group was markedly elevated to 68.6%, 79.3%, and 99.6% of the CN group, respectively. (Figures 5(c) and 5(d)).

3.6. Mel Reduced Pyroptosis of Testes Induced by CP. To explore whether pyroptosis is involved in CP-induced spermatogenesis dysfunction, mRNA levels of *GSDMD*, *GSDME*, *IL18*, *IL1b*, and *Caspase1* were assessed by qPCR. We found that CP treatment significantly increased the *GSDMD*, *IL18*, and *Caspase1* mRNA levels to 3.83-, 2.20-, and 1.60-fold of CN group, respectively. Pretreatment with Mel suppressed the CP-induced elevations in *GSDMD* and *IL18* expression to 1.51- and 1.73-fold of the CN group, respectively (Figure 6(a)). Western blot was used to confirm the protein level of GSDMD and IL18. The GSDMD and IL18 levels in the CP group were significantly increased to 225.8% and 135.8% of the CN group, respectively (Figure 6(b)). Mel given in combination with CP counteracted the increase of GSDMD and IL18, decreasing them to similar levels to those of the control (120.6% and 103.8% of the CN group).

4. Discussion

CP is an alkylating drug with antitumor and immunosuppressive properties and therefore widely used in clinical practice. However, many studies have shown that CP has widespread side effects because of its cytotoxicity [4]. Because of the high mitotic activity of spermatogenic cells, they are susceptible to damage induced by cytotoxic agents.

Mel, which is secreted from the pineal glands, is generally considered a multitasking molecule. As adjuvant chemotherapy, Mel strengthens chemotherapy-induced toxicity in cancer cells by inducing apoptosis, oxidative stress, and mitochondrial dysfunction [23]. On the other hand, Mel has a protective effect on normal cells by decreasing inflammation, apoptosis, and oxidative stress, as well as modulating sexual hormones and mitochondrial function [24]. Mel has been reported to reduce reproductive damage caused by certain chemotherapy drugs, including cisplatin, busulfan, and paclitaxel [19, 25, 26]. In this study, we aimed to determine the effect of CP on

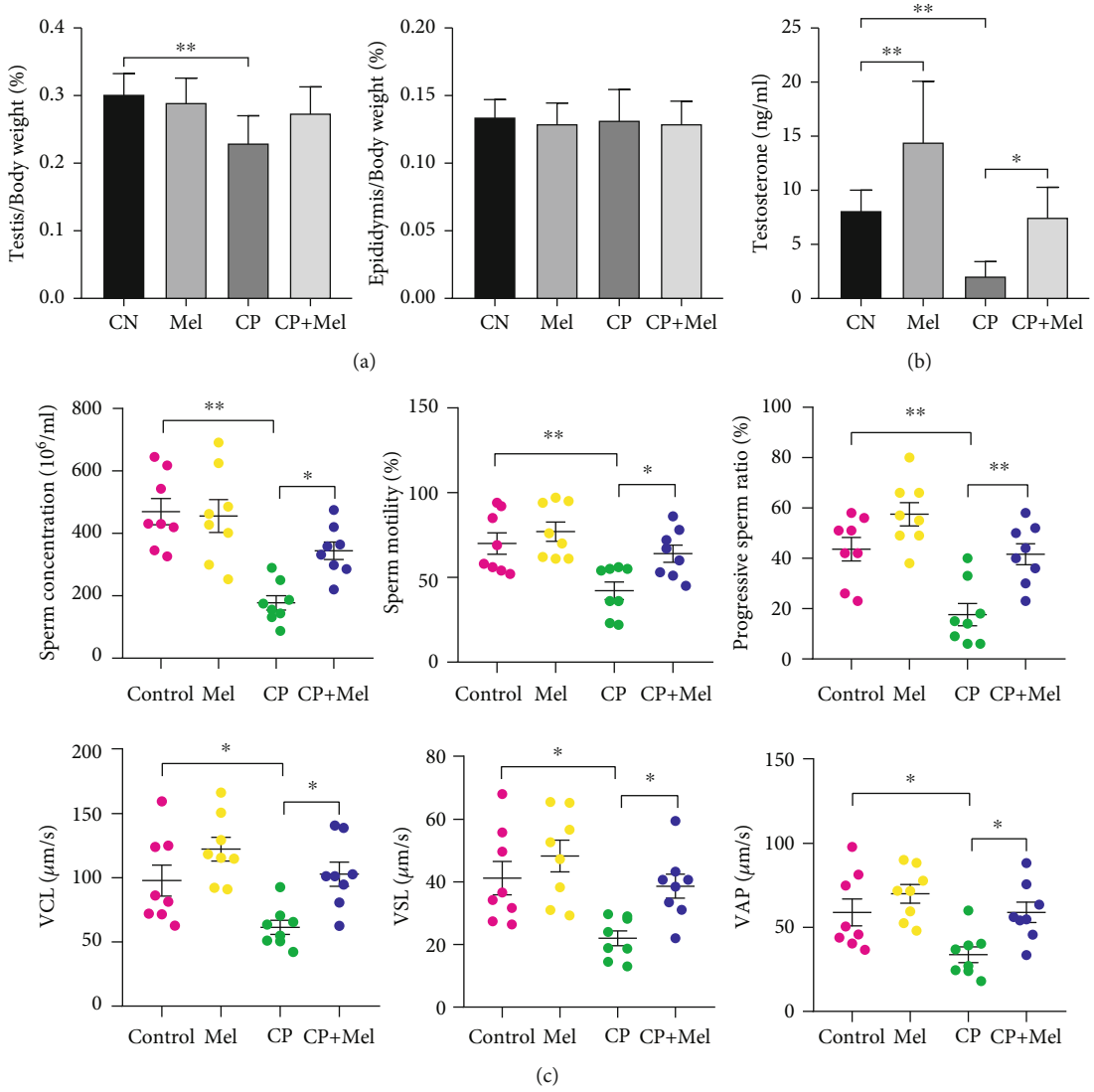

Figure 2: Mel treatment rescued CP-induced impairment in serum testosterone level and sperm motility. (a) Testicular and epididymal weights of four groups. (b) The testosterone concentration in blood serum. (c) Characteristics of sperm quantity and quality. Sperm concentration, sperm motility, progressive sperm ratio, straight-line velocity (VSL), curvilinear velocity (VCL), and average path velocity (VAP) were compared among four groups. $^{*}p < 0.05$; $^{**}p < 0.01$.

spermatogenesis and the protective mechanism of Mel against reproductive damage induced by CP.

The spermatogenic cycle, which includes meiosis and spermiogenesis, takes 35 days in mice [27]. In the present study, we chose a 6-week period after CP treatment to evaluate the effects on the spermatogenic cycle. We found reduced testicular weight when compared with the control, which was in accordance with previous reports [28, 29]. However, the reduction in testicular weight was compensated by self-repairing by week 6.

Mammalian spermatogenesis requires a period of amplification of cell numbers, reduction division to haploid cells, and the morphological transformation from haploid cells to spermatozoa, which are the processes of proliferation, meiosis, and spermiogenesis. The end result of these processes is the production of large numbers of spermatozoa [30]. The proliferation of spermatogonia and the meiotic of spermatocytes were assessed by PCNA and SYCP3, respectively. Here, significantly downregulated expressions of PCNA and SYCP3 were observed in CP-treated mouse testes throughout the experiment course of 6 weeks. The expression of PCNA and SYCP3 in the CP group was most suppressed compared with that in CN group at two weeks post CP treatment. Therefore, we selected mice at two weeks post CP administration as a testicular injury model for further in-depth study.

Histopathologically, CP reduced the thickness of seminiferous epithelium and induced seminiferous tubule atrophy. There was vacuolization of spermatogonia along the seminiferous epithelium, and part of elongated spermatids was shed. The number of Leydig cells in the interstitial space was also reduced. These results were in agreement with previous studies [3, 11, 28]. Testicular Leydig cells produced testosterone, one of the most important steroid hormones. Morphological analysis indicated that testicular

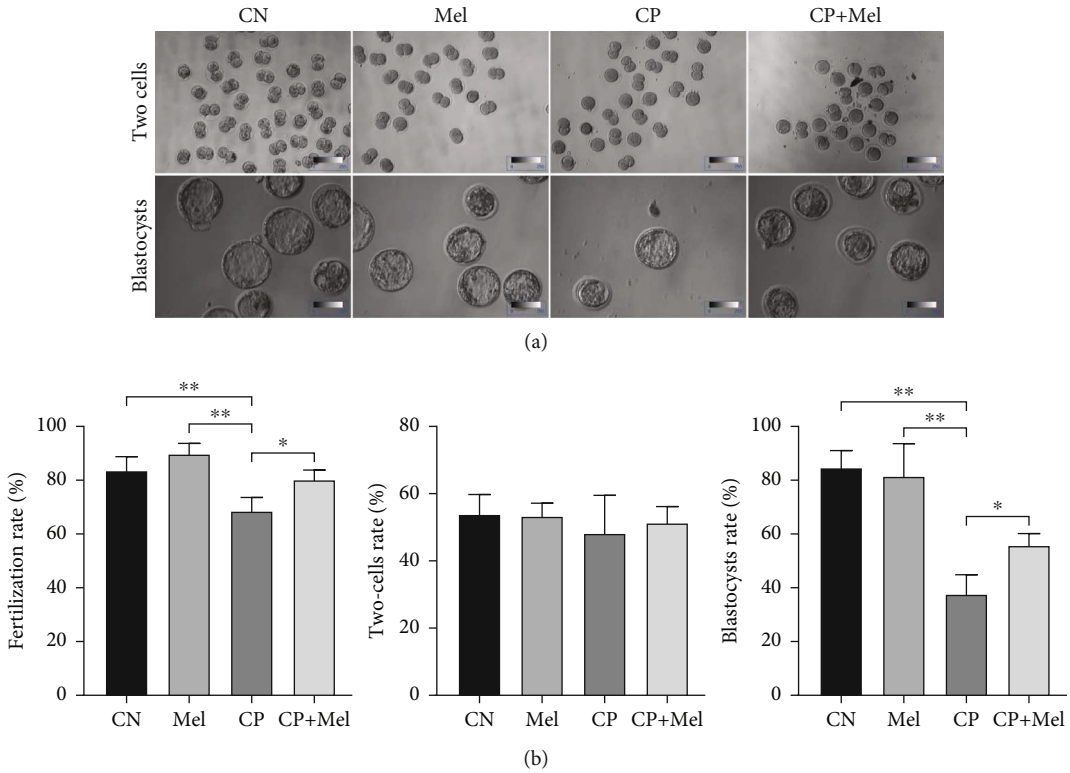

Figure 3: The impact of CP and Mel on male fertility in treated mice in an IVF experiment. (a) Representational images of two-cell embryos and blastocyst embryos that indicated oosperm development. (b) Statistical analysis of fertilization rate, two-cell embryo development rate, and blastocyst development rate. All values were shown as mean ± SD, $^{*}p < 0.05$, and $^{**}p < 0.01$.

structures were affected after CP treatment, which might induce functional disorder of the Leydig cells and cause the decline of serum testosterone level. Moreover, the reason for testicular weight reduction might be seminiferous tubule atrophy and testosterone decrease.

Testosterone is recognized as a key factor in the regulation of male sexual response, acting at both central and peripheral levels [31]. Testosterone is crucial for the progression of spermatogenesis and is active in the vital process of spermatogenesis, such as germ cell proliferation, meiosis, and the blood-testis barrier [32]. It also promotes sperm production in the testis. In this study, we observed reduced serum testosterone levels in CP-treated mice, which indicated that CP inhibited androgen synthesis. The decline in testosterone levels might be attributed to the reduced ability of Leydig cells to respond to luteinizing hormone or to direct suppression of testosterone synthesis.

Sperm count and motility are essential factors to measure semen quality. Mel effectively protected sperm function against CP-induced testis damage by improving sperm concentration and motility. It was reported that Mel receptors were present in the epididymis and that low-affinity melatonin-binding sites were found on spermatozoa [12, 33]. Thus, it was possible that Mel might affect sperm motility during the passage of spermatozoa through the epididymis. The protective effect of Mel on sperm mitochondria might be responsible for the improved sperm motility. As a known powerful antioxidant, Mel can stimulate mitochondrial respiration and ATP synthesis and increase the activity of respiratory chain complexes I and IV [34]. Earlier studies had demonstrated that Mel supplementation increased the concentration and motility of sperm by protecting sperm against the activation of the apoptotic pathway [35, 36].

In order to study the beneficial influence of Mel on sperm function disrupted by CP, we assessed male mouse fertility by IVF. We found decreased fertilization rate and blastocyst development rate in the CP group with an unaltered two-cell embryo development rate. The blastocyst development was negatively influenced by the inherent defect of sperm [37]. The paternal genome was activated after the two-cell stage, and the activation of the zygotic genome did not take place until this time [38].

IVF experiments showed that spermatozoa from CP-treated mice were inefficient in producing blastocysts, and Mel ameliorated sperm function by improving fertilization and embryonic development abilities. As mentioned above, Mel improved sperm concentration and progressive sperm ratio, which might be the reason that Mel enhanced the fertilization rate. The blastocyst development rate increased by Mel might result from reduced DNA damage in sperm. Mammalian spermatozoa are vulnerable to reactive oxygen species (ROS) attack due to that they are rich in polyunsaturated fatty acids. Many studies have proved that CP sensitizes spermatogenic cells to oxidative damage by decreasing superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and reduced glutathione (GSH) levels

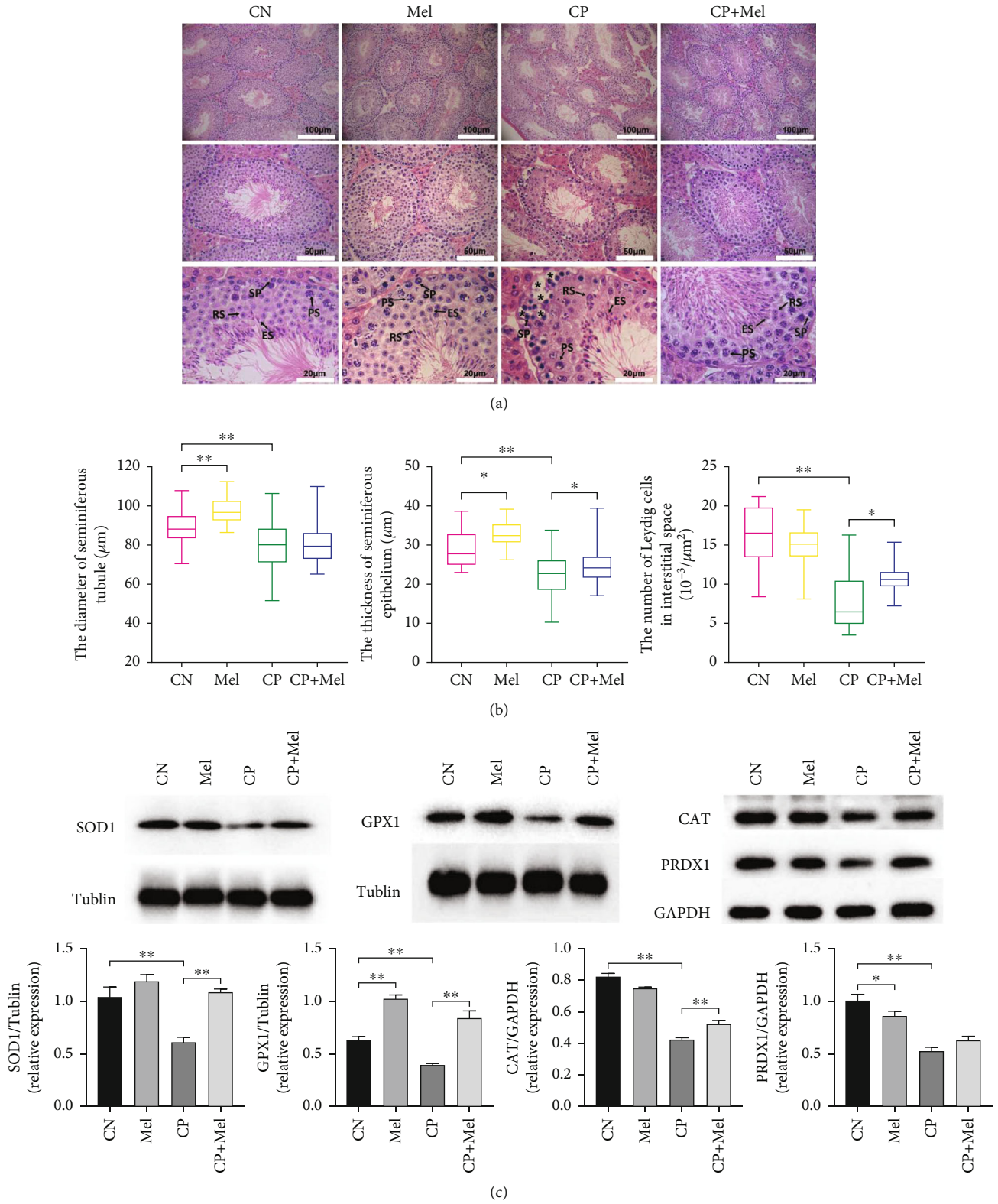

Figure 4: Histological changes and oxidative stress in the testes induced by the Mel and CP treatments. (a) The seminiferous tubules of CP-treated mice showed vacuolization and atrophy compared with the CN and Mel groups, which was restored by Mel cotreatment with the normal cellular content in seminiferous tubules. SP: spermatogonia; PS: pachytene spermatocytes; RS: round spermatids; ES: elongated spermatids. * showed vacuolization in the seminiferous epithelium. The vacuolization in the seminiferous epithelium was shown as pentagonal plum blossom, not hexagonal plum blossom. (b) The diameter of the seminiferous tubule, the thickness of the seminiferous epithelium, and the number of Leydig cells in interstitial space were analyzed by HE photographs. (c) Western blot of oxidative stress proteins in testes. $^{*}p < 0.05$; $^{**}p < 0.01$.

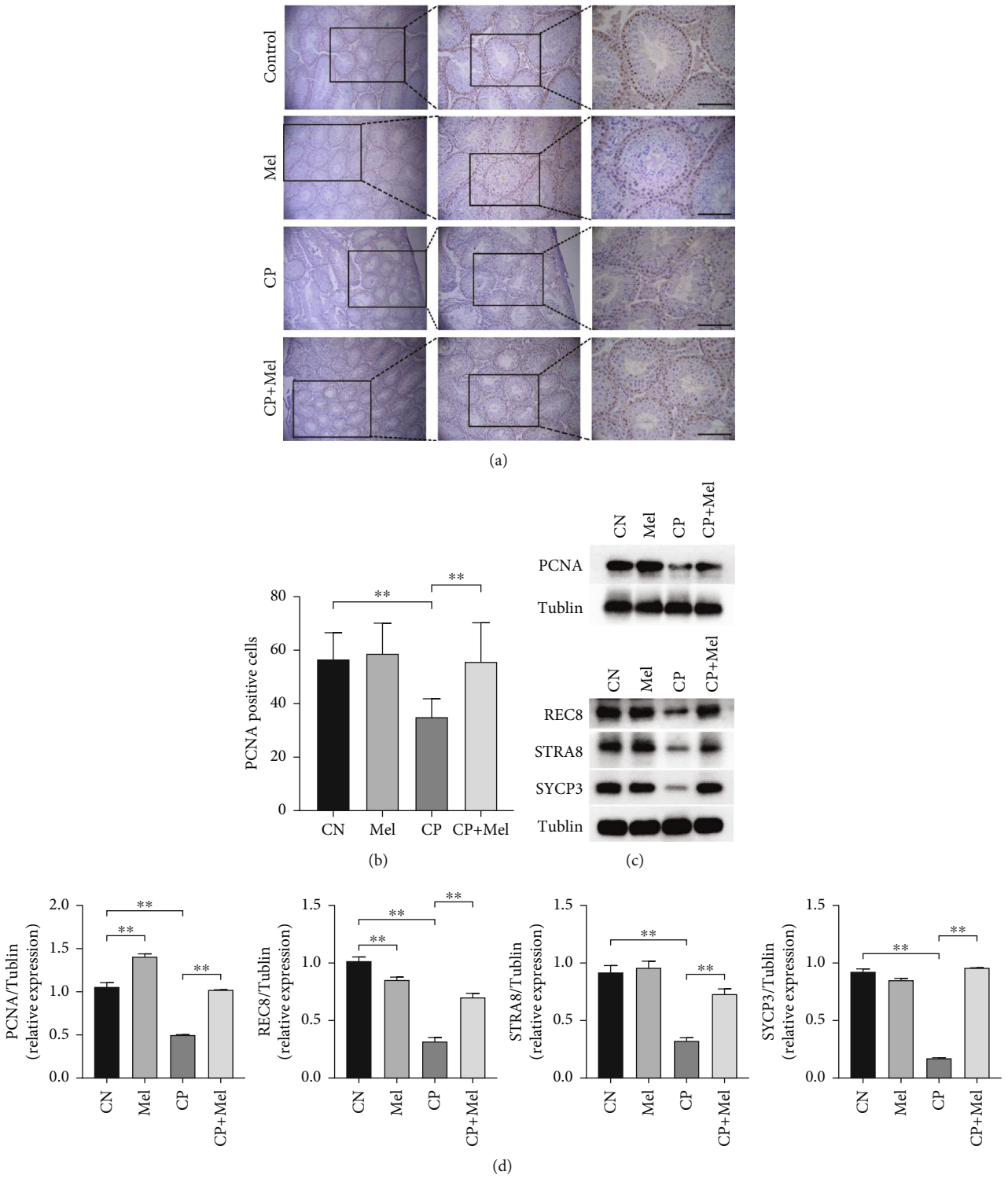

Figure 5: Mel promoted the proliferation and meiosis of germ cells which were damaged by CP. (a) IHC was carried out against PCNA to visualize spermatogenic cells. PCNA was mainly found in the nucleus of spermatogonia. The scale bar represented 50 μm. (b) The PCNA-positive cells were statistically analyzed under ten fields of each group. (c) Western blotting was carried out against proliferation-related protein PCNA and meiosis-related proteins REC8, STRA8, and SYCP3. (d) The relative integrated density of the bands of PCNA, REC8, STRA8, and SYCP3. Results were expressed as mean ± SD, $^{*}p < 0.05$, and $^{**}p < 0.01$.

[11, 39]. CP induced oxidative stress in the testis, and the ROS caused DNA damage in the spermatozoa. As a certified ROS scavenger, Mel can directly scavenge peroxyl radicals, singlet oxygen, and superoxide anion radicals [40]. Our results suggested that the capacity of germ cells to eliminate the excessive production of ROS was restored after Mel

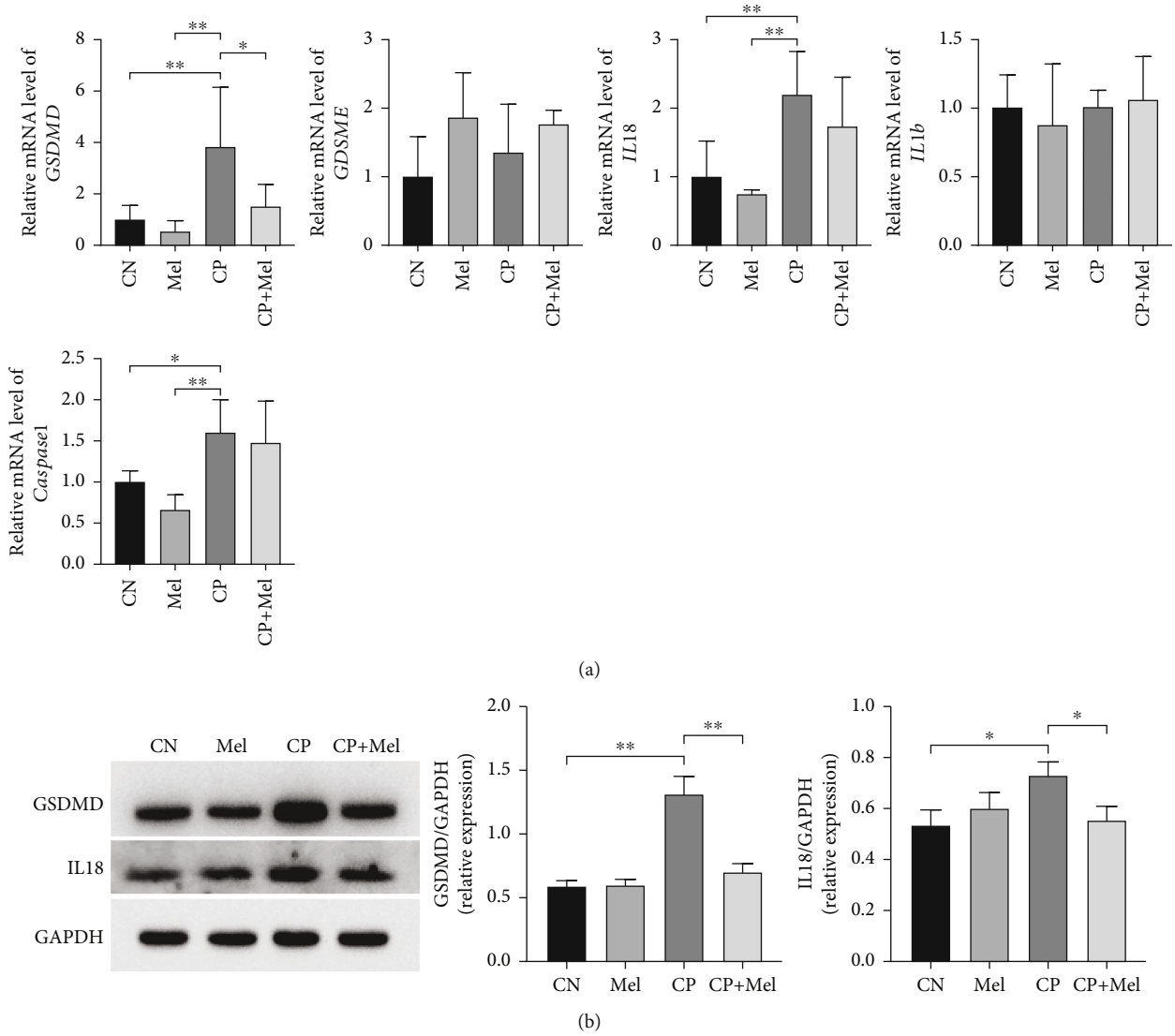

Figure 6: Analysis of pyroptosis-related proteins in CP and Mel-treated mice. (a) qPCR results showed the mRNA expression levels of pyroptosis-related gene (*GSDMD*, *GSDME*, *IL1b*, *IL18*, and *Caspase1*) in four groups. (b) Representative protein bands and statistical analysis of GSDMD and IL18. Data were presented as mean ± SD, $^{*}p < 0.05$, and $^{**}p < 0.01$.

treatment. Mel alleviated CP-induced oxidative stress damage and restored blastocyst development rate by reducing DNA damage in sperm.

Besides, research has shown that Mel plays an active role in oocyte maturation, fertilization, and embryonic development. The number of mature oocytes, the fertilization rate, and the number of high-quality embryos were increased after supplementation with Mel [41, 42]. These studies showed that Mel had an important role in reproductive functions and had a promising prospect for clinical use.

An increase in oxidative stress makes sperm DNA susceptible to denaturation and fragmentation, which probably play an important role in the induction of sperm abnormalities [43]. As a result, we examined the effects of CP on spermatogonia proliferation, meiosis, and pyroptosis induced by DNA damage. PCNA is a well-recognized biomarker associated with cell proliferation. The decrease in PCNA-positive germ cell numbers and expression levels indicated that CP significantly affected the proliferation of spermatogonia, which might be the reason of reduction in sperm concentration and implicate an effect on subsequent meiosis. SYCP3 is a vital protein that participated in synapsis, recombination, and segregation of meiotic chromosomes, in which mutations are associated with male azoospermia. As a crucial component of the meiotic cohesion complex, REC8 can regulate sister chromatid cohesion and recombination between homologous chromosomes. STRA8 is involved in the regulation of meiotic initiation in both spermatogenesis and oogenesis. The different expression levels of meiosis marker molecules indicated that CP impaired the meiosis of spermatocytes, thereby affecting sperm production and quality. Meanwhile, Mel improved the proliferation and meiosis of germ cells which were impaired by CP. It is reported that paclitaxel or doxorubicin reduced the expression of

proliferation- and meiosis-related proteins in germ cells, and this effect could be partially improved with Mel administration [26, 44]. Moreover, adding Mel to the freezing medium can effectively promote the proliferation and differentiation of spermatogonial stem cells (SSCs) after transplantation in an azoospermia mouse model [45]. The protective effect of Mel on proliferation and meiosis against CP damage might be due to reducing ROS production, increasing the SSC viability, and promoting the proliferation and differentiation of SSCs.

GSDMD is cleaved by activated caspase-1 and induces caspase-1-dependent pyroptosis as an implementing protein. It is reported that CP induced ovarian injury with many features of pyroptosis, such as increase of IL1β, IL18, and GSDMD [46]. Exposure to CP caused toxicity in the ovary, muscle, and hepatocytes by inducing pyroptosis [6, 8, 46], but no research has shown that CP caused testis pyroptosis. Here, we demonstrated that CP treatment increased the expression of *GSDMD*, *IL18*, and *Caspase-1* at the transcriptional level in mice, which were reduced after Mel supplementation. Moreover, the protein expression level of GSDMD and IL18 was consistent with the mRNA level. Endogenous and exogenous factors have been reported to cause NOD-like receptor family pyrin domain-containing 3 (NLRP3) to bind to pro-caspase-1 and subsequent cleaved caspase-1. The activated caspase-1 cleaves GSDMD protein and promotes the release of IL1β and IL18, which induce pyroptosis and inflammatory reactions. [47]. Our results showed that CP induced caspase-1 upregulation, and activated caspase-1 promotes GSDMD activation and IL18 release, eventually leading to the decline of testis function. It is reported that Mel attenuated adipocyte pyroptosis by inhibiting GSDMD transcription. Mel also alleviated lipopolysaccharides- or obesity-induced NLRP3 inflammasome activation and pyroptosis [48]. Our results demonstrated that Mel alleviates CP-induced pyroptosis by downregulationing of GSDMD and IL18 in mouse testis. The reduction of GSDMD activation and IL18 release might be the reason that Mel inhibited pyroptosis of the testis. Therefore, antipyroptosis effect was an important role of Mel in protecting the testis from CP damage.

To our best knowledge, the current study provided another molecular mechanism of Mel in ameliorating testicular damage induced by CP. The molecular mechanisms of Mel were mainly focused on its ability to ameliorate oxidative stress in previous studies. Intriguingly, our results indicated that CP induced pyroptosis in germ cells, and Mel alleviated CP-induced spermatogenesis disorder and restored fertility by preventing pyroptosis in a mouse model. However, the cellular signaling pathway of pyroptosis has not been detected. Moreover, this mechanism still awaits confirmation in cellular models. In our future study, we will explore the function of Mel in preventing pyroptosis and the underlying signaling pathway and molecular mechanism using type A spermatogonial stem cells (SSCs, C18-4), type B SSCs (GC-1), and Leydig cells (TM3).

In conclusion, Mel has the potential to protect male fertility in chemotherapy patients. In addition, cell pyroptosis may play a prominent role in testicular toxicity associated with chemotherapeutic agents. Targeted modulation of testicular pyroptosis is a new direction for fertility protection for tumor patients in the future.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

There are no known conflicts of interests that could affect the work reported in this paper by the authors' declaration.

Authors' Contributions

Fujun Liu and Xuexia Liu conceived and designed the experiments. Zhan Song, Jiahui Wang, Peng Zhu, and Zhixin Wang performed the experiment and data analysis. Zhan Song and Jiahui Wang wrote the manuscript. Fujun Liu, Zhan Song, and Jiahui Wang contributed to funding support. Zhan Song and Jiahui Wang contributed equally to this work.

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